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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The epidermal growth factor receptor (EGFR) is an important mediator of breast cancer tumorigenesis and metastasis. While much is known about EGFR signal transduction related to its tyrosine kinase activity, less is known about the protein tyrosine phosphatases (PTPs) which must be present to modulate the cellular effects of the EGFR by dephosphorylating the receptor and its substrates. Evidence derived from several approaches suggests that the transmembrane PTP LAR may be involved in EGFR signaling in mammary gland development and tumorigenesis. The *hypothesis to be tested in this proposal is that LAR plays an important role in EGFR-dependent mammary gland development and tumorigenesis through negative modulation of EGFR signal transduction.* Work in the first year has supported this hypothesis. EGF receptor signaling was shown to be increased in SV40-immortalized cells from $LAR^{-/-}$ knockout mice. Cell density influenced the magnitude of the effect. LAR expression is also shown to be regulated by cell density, with concentrations increasing markedly as cell density increases. Functional E-cadherin complexes are necessary for this effect. It is intriguing to speculate that the density dependent increase in LAR is involved in the suppression of EGF signaling. Finally, we have now developed a transgenic mouse with targeted expression of human LAR to the mammary gland. Characterization of mammary gland development and tendency to tumorigenesis will be pursued. This will be an important complement to our parellel studies of development and tumorigenesis in the LAR knockout mouse.

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INTRODUCTION

The epidermal growth factor receptor (EGFR) and the related protein erbB2 have been implicated as important mediators of breast cancer tumorigenesis and metastasis. While much is known about EGFR signal transduction related to its tyrosine kinase activity, less is known about the protein tyrosine phosphatases (PTPs) which must be present to modulate the cellular effects of the EGFR by dephosphorylating the receptor and its substrates. Evidence derived from several approaches suggests that the transmembrane PTP LAR may be involved in EGFR signaling in mammary gland development and tumorigenesis. Two sets of data are particularly important. First, the LAR knockout mouse has been shown to have a defect in terminal mammary gland development. Second, we have shown that suppression of cellular LAR by 60% using an antisense expression vector results in a 3-4 fold elevation of EGF-dependent receptor signaling. Based upon these and other observations, the *hypothesis to be tested in this proposal is that LAR plays an important role in EGFR-dependent mammary gland development and tumorigenesis through negative modulation of EGFR signal transduction.*

ANNUAL REPORT

The research question (hypothesis) to be tested in this proposal is that *LAR plays an important role in EGFR-dependent breast cancer through negative modulation of EGFR signal transduction*. With the long term goal of understanding the mechanisms by which EGF signaling is abnormal in breast cancer, two specific aims are being pursued: **First**: characterize the impact of LAR on TGFα-dependent abnormal mammary gland development. This requires a mouse model which has the combined characteristics of a LAR deficient (knockout) mouse and a mouse which has a strong tendency to abnormal mammary development and tumors because of increased expression of the tumor-promoting gene (TGFα) in the mammary gland. The hypothesis would predict that abnormal mammary development will be more pronounced in those mice with the absence of the modulatory effects of LAR. **Second**: define the mechanism by which LAR mediates its modulatory effect on EGFR signaling and alters mammary gland development. Examine how LAR interacts with EGFR. Is it direct or through some intermediate steps?

Aim #1: Characterize the influence of PTP LAR on TGFα-dependent abnormal mammary gland development.

This objective requires the crossing of LAR knockout mouse with mice expressing TGFα targeted to the mammary epithelium. These studies require the monitoring of the mice for tumor formation for a period of 12 months and beyond. Early in the funding year, it became known that the co-investigator, Dr. William Kisseberth, was relocating to the Dept. of Clinical Sciences, College of Veterinary Medicine, Ohio State University. While this relocation will not alter the objectives of this study, Dr. Kisseberth's contribution to the project included this aspect of the study, ie. the crossbreeding of the mice and the monitoring of same for tumor formation. Thus, it was decided to postpone the initiation of the mouse crossing studies until after the relocation so that they can be completed at Ohio State. Relocation of the animals during their tumor latency was not considered to be wise.

In place of the crossbreeding studies, we were able to pursue the development of a transgenic mouse with targeted expression of the human LAR gene to the mammary gland. The development of this transgenic animal did not require funds from this grant. Dr. Kisseberth's department provided support for this aspect of the study. Notheless, this transgenic model will be invaluable in pursuing the objectives of this project, that is, the role played by LAR in regulating EGF receptor signaling and the impact of this on mammary gland development and tumorigenisis.

The steps in this process were as follows:

- Obtained MMTV cassette in a pSP73 vector from Dr. Kai-Shun Chen at the University of Wisconsin
- Obtained the human LAR gene in the pSP6 vector from Dr. Michel Streuli of the Dana Farber Cancer Institute
- Subcloned the human LAR cDNA minus the polyA sequence (a 6.5 kb fragment) into the MMTV cassette

- Excised and purified the MMTV-LAR cassette (11.2 kb) from the pSP73 vector
- The linear MMTV-LAR construct was injected into mouse blastocysts and placed in a recipient mice.
- Six founder lines have been obtained as determined by detection of SV40 poly A sequence by PCR within the mouse genome of F1 offspring.
- Human LAR message has been detected in the mammary glands of all founder lines using a PCR assay that discriminates between mouse and human LAR message.

These transgenic mice are able to breed normally and nurse their young. Thus, no obvious phenotype is affecting the mammary gland. Pathologic studies are underway to characterize mammary gland development during pregnancy and lactation in each of the mouse lines. Subsequent investigations will include the characterization of EGF receptor signaling in isolated mammary epithelial cells from the LAR overexpressors and compared to controls. In summary, though the crossbreeding of LAR knockouts to TNFα overexpressors is delayed until Year 2, the generation of a transgenic mouse with targeted expression of LAR to the mammary epithelial cells is a very important new model in which to test our hypothesis that the PTP LAR is an important regulator of EGF receptor signaling in the mammary gland.

Aim 2: Elucidate the mechanism by which LAR modulates EGFR signaling in the mammary gland.

Two aspects of Aim #2 have shown progress during Year #1. These include **1)** the investigation of potential indirect effects of LAR on the EGFR-mediated by LAR- E-cadherin interactions and **2)** the investigation of EGF receptor signaling in the LAR knockout mouse.

1) Expression of PTP LAR is Regulated by Cell Density Through Functional E-Cadherin Complexes: We have pursued an investigation of the mechanism by which cell density regulates LAR levels. We are testing the hypothesis that functional E-cadherin complexes regulate cellular LAR levels. It is known that E-cadherin also regulates EGF receptor signaling, with functional E-cadherin complexes being inhibitory to the mitogenic effects of EGF receptors. Ultimately, we are asking the question whether E-cadherin is inhibiting EGF receptor signaling by increasing levels of the potential EGF receptor phosphatase, LAR. The results of this investigation are being organized into a manuscript. The Abstract, Results, and Figures from this manuscript are included below.

Abstract: The receptor-like protein tyrosine phosphatase LAR has been implicated in receptor tyrosine kinase signaling pathways while also displaying cell density dependency and association with cadherin complexes. While physiologic substrates for LAR have not been unequivocally identified, β-catenin associates with LAR and is an *in vitro* substrate. With the implication that LAR may play a role in regulating E-cadherin

dependent cell-cell communication and contact inhibition, the relationship of LAR to E-cadherin was investigated.

LAR expression increased with cell density in the human breast cancer cell MCF-7 and in Ln 3 cells derived from the 13672NF rat mammary adenocarcinoma. LAR protein levels rapidly decreased when cells were replated at low density after attaining high expression of LAR at high cell density. COS-7 cells displayed density dependent regulation of LAR expression when transiently expressing exogenous LAR under the control of a constitutively active promoter. Disrupting homophilic E-cadherin complexes by chelating extracellular calcium caused marked decreases in LAR protein levels. Similarly, blocking E-cadherin interactions with saturating amounts of E-cadherin antibody (HECD-1) also led to a rapid and pronounced loss of cellular LAR. Finally, mimicking cell surface E-cadherin engagement by plating cells on dishes coated with HECD-1 resulted in a 2 fold increase in LAR compared to controls. These results suggest that density-dependent regulation of LAR expression is mediated by functional cadherin complexes by a mechanism that does not require regulation of LAR transcription.

Results: *LAR protein levels change with cell density in parallel with changes in E-cadherin.* Our previous studies indicated that LAR protein expression is cell density dependent (). In light of the observed association of LAR with constituents of the adherens junctions and the defect in terminal differentiation of the mammary gland in LAR knockout mice, the current study was initiated to explore the mechanism of cell density dependency of LAR using breast cancer cell lines. LAR protein levels were examined in rat mammary adenocarcinoma cell lines (MTC, Ln 3), as well as in the human MCF-7 breast carcinoma cells. Each cell type was grown for several passages at either high (85-100%) or low (10-20%) cell density. Cell lysates were then normalized for protein and separated on SDS-PAGE gels. In the human MCF-7 cell line, LAR protein increased markedly from low to high density as detected by Western blotting using an antibody to the LAR extracellular or E-subunit (figure 1A). It was possible that the difference in LAR E-subunit expression did not reflect expression of the catalytically active P-subunit. For example, loss of the E-subunit alone may have been due to proteolytic cleavage or shedding of the extracellular domain at the lower density (). When MCF-7 lysates were probed with an antibody to the catalytic or P-subunit of LAR, comparable density-dependent changes in LAR expression were observed (figure 1B). Similarly, the highly metastatic Ln 3 cells, derived from the 13762NF rat mammary adenocarcinoma, exhibited a large increase in LAR protein from low to high density when detected with the P-subunit antibody (figure 1C). Interestingly, the non-metastatic MTC cells (from the same tumor), did not express LAR (figure 1C). The less metastatic Ln 2 cells, also derived from the 13762NF tumor, showed a progressive increase in LAR levels from low to high density similar to the Ln 3 (data not shown). Analysis of E-cadherin levels as a function of cell density paralleled LAR levels in each of the cell lines (data not shown). Curiously, MTC cells, which expressed little LAR, did not express detectable levels of E-cadherin.

EGF receptor, erb-B2, and src kinase inhibitors and LAR expression. As stated previously, LAR has been implicated in several tyrosine kinase signaling pathways. It

has been reported that LAR protein expression increased when breast epithelial cells were transformed by the neu oncogene (31). In the present study, the role of EGF receptor, erbB2, and src kinase signaling in regulating LAR expression was examined. MCF-7 cells were grown to a density nearing confluence before being treated with inhibitors to the EGF receptor (Compound 56), erbB2 (AG 825), or src kinase (PP1), or were treated with a general kinase inhibitor (geldanamycin). No significant changes in morphology were seen after overnight treatments in MCF-7 cells. The only effect was a slight decrease in LAR protein with the non-specific tyrosine kinase inhibitor, geldanamycin, in the MCF-7 cells (figure 2A). The inhibitors were also tested in the Ln 3 cell line with similar results (figure 2B, 2C and data not shown). Inhibition of EGF receptor autophosphorylation by compound 56 was confirmed as shown in figure 2C. Thus, these kinase signaling pathways have no effect on LAR levels under the conditions studied. This result was somewhat surprising because there is evidence to suggest that tyrosine signaling is modulated by density and these tyrosine kinase signals are thought to control the cellular response to density. Nonetheless, our data indicate that EGF receptor, erbB2, and src kinase signaling are not required for the high density-dependent maintenance of increased LAR protein levels in these cell lines.

LAR levels respond to rapid changes in cell density. Cell growth to high density is associated with higher levels of LAR. The following investigation examined the impact of acute changes in cell density on LAR expression. Cells were grown to confluence before being dissociated, pooled and replated at either high or low density. Within 4 hr of replating, LAR protein levels decreased markedly when replated at low density. In contrast, replating at high density preserved the pre-existing LAR expression (figure 3A,C,D). E-cadherin levels decreased in parallel with those of LAR when cells were exposed to low cell density conditions (figure 3B). The decreased expression of LAR reflected changes both in the E and P subunits. While the extracellular E subunit potentially could have been influenced by shedding, the similar density-dependent changes in LAR observed in LN3 cells (figure 3C,D) probed with either a P-subunit or E-subunit antibody argue that decreased cell density initiated the loss of total cellular LAR protein.

The above investigation demonstrated that LAR levels decreased markedly within 4 hr of an acute decrease in cell density. Next the response to an acute increase in cell density was investigated. Cells were maintained at 10-30% confluence for several days before harvesting and replating at high density. LAR protein expression was analyzed by Western blotting at the times indicated (figure 4A). Controls were undisturbed cells which were maintained at high density during the 48 hr experiment. LAR was modestly increased within 8 hr of replating at high density and continued to increase for 48 hr. By 24-48 hours, the level of LAR protein reached that of the high density control cells. Because the MCF-7 cells do not proliferate rapidly and the cells had been plated at high density, the increase in LAR protein expression is not likely due to cell proliferation. Interestingly, the increased LAR protein expression paralleled the formation of cell-cell contacts. As observed by light microscopy, the MCF-7 cells began to spread at 4-6 hours and visible cell-cell contacts were observed at 8-10 hours. Increased LAR protein also correlated with an increase in E-cadherin protein in these cells (figure 4B). From these

data, we hypothesize that LAR protein levels increase with the formation of functional cell-cell contacts.

Density dependent changes in LAR levels do not require changes in the synthetic rate. To address the possibility that the difference in LAR levels at varying densities was due to differing rates of transcription, we used a method similar to that of M. Gebbink et. al. (). COS-7 cells were transiently transfected with LAR cDNA under the control of a constitutively active promoter. The transfected cells were incubated for 24 hours, dissociated and replated at either high (> 85%) or low (< 30%) density. The cells were lysed 24 hours later and LAR expression was analyzed. Since exogenous LAR expression is under the control of a constitutively active promoter, the transcription rate of LAR remains constant whether cells are at high or low densities. Changes in LAR levels would be due to altered rates of protein degradation or mRNA stability. As shown in figure 5, LAR expression in transfected COS cells, which express no detectable endogenous LAR, increased several fold with increasing cell density (figure 5).

LAR protein levels are altered by disruption of E-cadherin mediated cell-cell contact. We hypothesized that cell density dependent increases in LAR protein expression were due to a signal mediated by E-cadherin dependent cell-cell contact. To pursue this possibility, MCF-7 cells were treated with EGTA to disrupt cell-cell contact through chelation of the extracellular calcium required for E-cadherin homophilic binding between cells. Following EGTA treatment, the cells were examined by light microscopy to document morphological changes. By 1 hr of treatment with EGTA, MCF-7 cells became rounded and detached from one another. At 6 hr, MCF-7 cells had no cell-cell contacts, but the cells remained attached to the culture dishes. By 18 hr, cells had begun to lift from the dish and marked cell death was apparent. Cells were lysed at these various time points (excluding 18 hr) and protein-normalized lysates were separated on SDS-PAGE gels. Western blotting for the LAR E-subunit revealed that LAR was expressed in high amounts at 0, 1, and 2 hr, but decreased sharply between 2 and 4 hr (figure 6A). After 6 hr, LAR protein was undetectable (data not shown). E-cadherin protein levels were also examined to determine whether loss of cell-cell contact induced by EGTA also caused changes in E-cadherin expression. E-cadherin protein was also high at 0, and 1 hr, but unlike LAR, began to decrease somewhat earlier between 1 and 2 hr (figure 6B). As a control for protein concentration, cell lysates were probed for β-catenin. There was no change in β-catenin protein expression at any time point (figure 6C). The correlation between LAR and E-cadherin expression is consistent with our earlier observations and raising the question of whether E-cadherin expression, which responds to changes in cell density, controls LAR expression.

Since expression levels of LAR and E-cadherin were observed to change in parallel, we sought to determine whether a more direct relationship between the two proteins could be established. The homophilic cadherin-mediated cell-cell interactions are the molecular sensors of cell density. Thus, we hypothesized that E-cadherin interactions may be responsible for the density dependent change in LAR protein levels. To determine whether functional cadherin complexes control density dependent LAR expression, we inhibited extracellular cadherin interactions directly by using an E-

cadherin-specific monoclonal blocking antibody, HECD-1. Confluent MCF-7 cells were treated with 1, 5, or 10 μg/ml of HECD-1 to inhibit cell-cell contact. When the cells were analyzed for morphological changes, light microscopy revealed that 5 and 10 μg/ml HECD-1 caused greater than half of the cells to round up (data not shown). When 1 μg/ml of antibody was used, the cells maintained their cell-cell adhesive properties. Western blotting showed a marked decrease in LAR expression when HECD-1 was added at 5 or 10 μg/ml as compared to the control (figure 7A). Thus, cadherin complexes mediate the expression of LAR protein. Under the same conditions, E-cadherin protein levels were not reduced, indicating that functional E-cadherin interactions rather than just cellular E-cadherin expression regulated LAR expression.

E-cadherin alone is not sufficient for the regulation of LAR. Since inhibition of E-cadherin homophilic interactions resulted in decreased LAR expression, we hypothesized that functional E-cadherin complexes were responsible for density-dependent regulation of LAR expression, perhaps by sequestering LAR at adherens junctions and decreasing its turnover. To examine this possibility, COS-7 cells were transiently co-transfected with mouse E-cadherin and human LAR. These cells were analyzed for the ability of exogenously expressed E-cadherin to increase the amount of exogenously expressed LAR. While the results were variable, it was concluded that exogenous E-cadherin did not consistently alter the exogenous expression of LAR in these cells (figure 8). Nevertheless, protein levels of exogenously expressed LAR continued to be regulated by cell density in the co-transfected COS-7 cells (figure 5), suggesting that E-cadherin alone is not sufficient to regulate LAR expression.

Activation of E-cadherin complexes is sufficient to increase LAR expression. Functional E-cadherin complexes appear to be involved in the regulation of LAR expression. Ectopic expression of E-cadherin may not have increased LAR levels if it did not appreciably increase the amount of functional E-cadherin complexes. To more directly investigate the role of functional E-cadherin complexes in regulation of LAR levels, MCF-7 cells which had been maintained at low density for a minimum of 48 hr were replated on culture dishes coated either with HECD-1 or rabbit IgG. Cells were plated at low density with little opportunity for cell-cell contact. LAR expression did not increase appreciably in control cells but increased at least 2 fold in cells plated on the anti-E-cadherin antibody.

Figures for LAR / E-Cadherin Study

Figure 1.

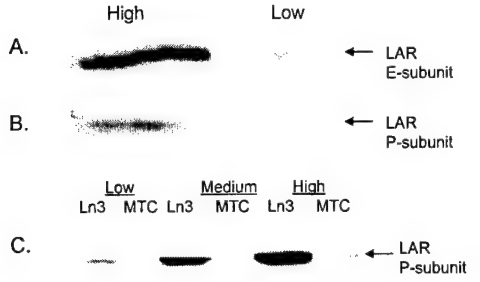


Figure 2

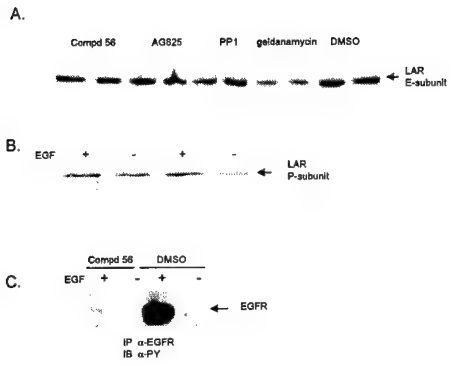


Figure 3

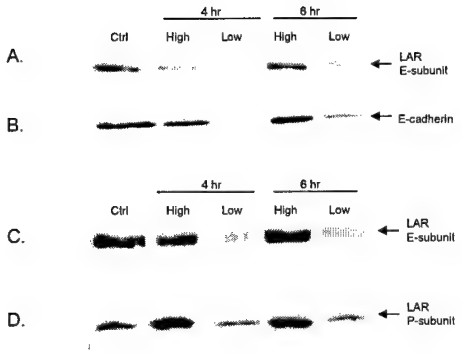


Figure 4

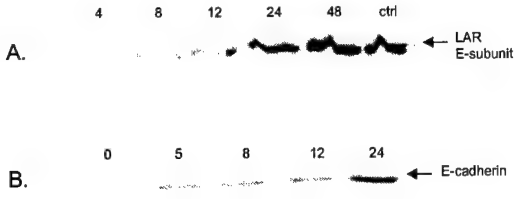


Figure 5

Figure 6

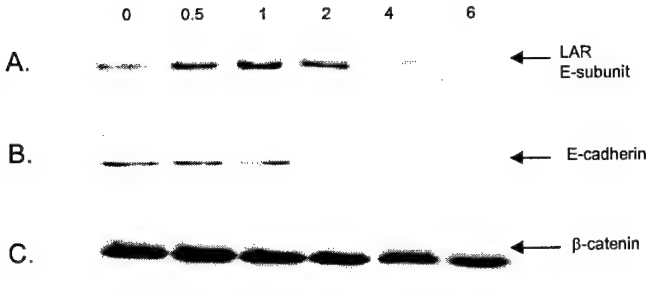


Figure 7

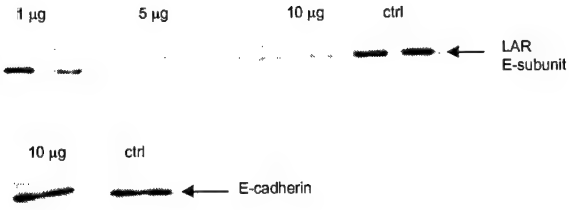


Figure 8

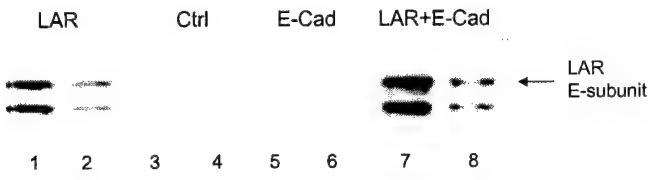


Figure 9

2). EGF receptor signaling in immortalized cells from $LAR^{+/+}$ and $LAR^{-/-}$ mice

To assess the effect of LAR deletion on EGF receptor signaling, populations of cells from $LAR^{+/+}$ and $LAR^{-/-}$ mice are being studied. Our first approach is to immortalize cells with temperature sensitive SV40 virus. We have first applied this approach to liver cells. We are currently developing the technique and establishing mammary epithelial cell lines. Below, immortalized hepatocyte populations from $LAR^{+/+}$ and $LAR^{-/-}$ mice were matched for EGF receptor content. EGF-dependent tyrosine phosphorylation was examined as a function of cell density and plating on fibronectin.

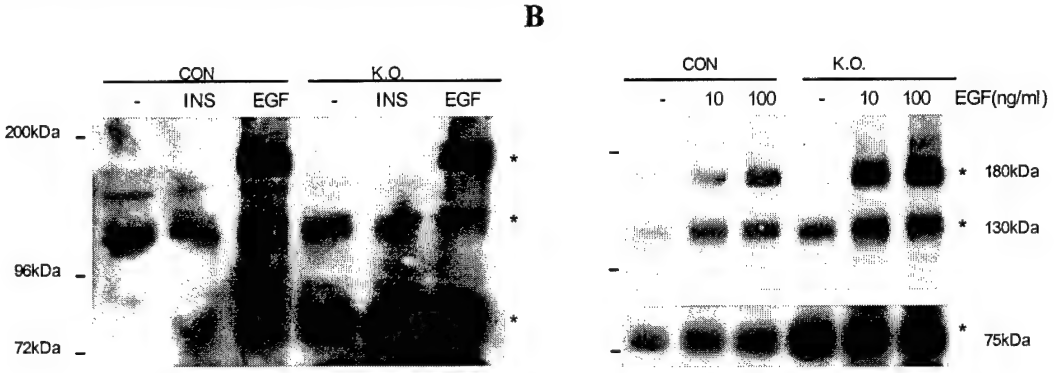
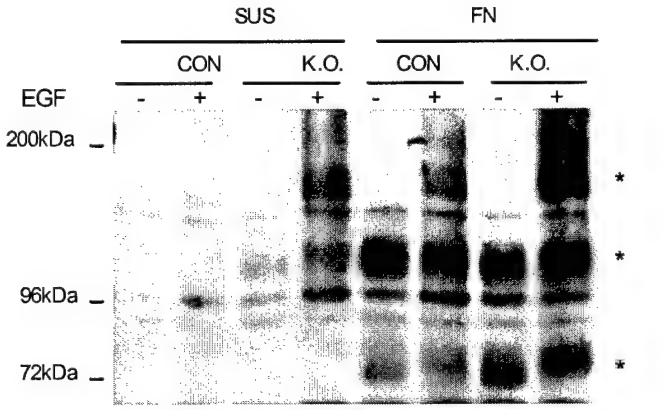


Figure 10. EGF-dependent tyrosine phosphorylation in SV40 transformed liver cells from $LAR^{-/-}$ and control mice. Cells were either grown to near confluence (A) or maintained at less than 30% confluence (B) prior to addition of EGF at 100ng/ml and insulin at 10^{-7}M for 1 min. Cells were lysed, immunoprecipitated with anti-PY antibody, separated by SDS-PAGE, and probed by Western blotting with anti-PY. Samples were normalized to protein before separation. Lower band in B is a darker exposure to enhance the 75kDa protein band. * indicates prominent tyrosine-phosphorylated bands.

Figure 11. EGF-dependent tyrosine phosphorylation in SV40 immortalized liver cells from $LAR^{-/-}$ and control mice. Cells were maintained in suspension for 1 hr before plating onto fibronectin for 30 min. EGF was added for the final 1 min. Cells were lysed, immunoprecipitated with anti-PY antibody, separated by SDS-PAGE, and probed by Western blotting with anti-PY.



The above data revealed no difference in EGF-dependent tyrosine phosphorylation between $LAR^{-/-}$ and control cells when grown to near confluence but a large increase in EGF-dependent tyrosine phosphorylation in $LAR^{-/-}$ cells when grown at low density. One prominent EGF-dependent protein was observed at approximately 180 kDa and is most likely the EGFR. Two prominent tyrosine phosphorylated proteins at approximately 130 kDa and 75 kDa were not markedly different with EGF. The 130kDa protein was very similar in intensity in both cell lines while the 75 kDa protein was more intense in the $LAR^{-/-}$ cells. The 75 kDa and 130 kDa proteins were similar in size to paxillin and FAK, respectively. If these identities are correct, both phosphoproteins should be adhesion dependent. To test this characteristic, the $LAR^{-/-}$ cells and controls were maintained in suspension for 1 hr before plating onto fibronectin or remaining in suspension for 30 min. EGF was added for the final 1 min. As shown in **Figure 11**, the putative EGFR was more highly phosphorylated in the adherent $LAR^{-/-}$ cells as was the 75 kDa protein. The 75 kDa protein was entirely absent in cells maintained in suspension while the 130 kDa protein was weak but detected in cells in suspension.

These immortalized liver cell studies indicate that LAR expression can affect growth factor receptor autophosphorylation. The absence of an effect in cells near confluence is somewhat surprising given our evidence that LAR expression increases with cell density as a function of E-cadherin. Nonetheless, the increased EGF receptor signaling in LAR negative cells indicates that LAR may well be an important regulator of EGF receptor signaling. We are continuing these investigations with primary and immortalized mammary epithelial cells.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that expression of the LAR phosphatase in the human breast cancer cell line, MCF7, is dependent upon cell-cell contact.
- Characterized the mechanism by which LAR protein is regulated and demonstrated that functional E-cadherin complexes are necessary and sufficient for this effect.
- This is particularly relevant to the objectives of this project because engagement of E-cadherin complexes are also known to exert an inhibitory effect on EGF receptor signaling, particularly mitogenesis. Might it be that the E-cadherin effect is, in part, mediated through the expression of the PTP LAR as our hypothesis would suggest?
- Demonstrated an increase in EGF receptor signaling in immortalized cells from $LAR^{-/-}$ mice when compared to $LAR^{+/+}$ controls.
- A transgenic mouse model has been developed which targets expression of human LAR selectively to the mammary gland via the MMTV promoter. Five founder lines are being characterized for levels of expression.

REPORTABLE OUTCOMES

Manuscript in preparation:

Symons, JR and Mooney, RA (2001) Expression of PTP LAR is regulated by cell density through functional E-cadherin complexes.

Mouse transgenic model:

Targeted expression of human LAR selectively to the mammary gland via the MMTV promoter.

CONCLUSIONS

Work in the first year has supported the hypothesis that LAR is a negative regulator of EGF receptor signaling. This has come from our use of immortalized hepatocytes from the knockout mice. It is very important to validate these observations in immortalized and primary mammary epithelial cells from this mouse model. This work is in progress. We have also characterized the regulation of cellular LAR expression and have revealed an important role for E-cadherin in this process. LAR is regulated by cell density, with concentrations increasing markedly as cell density increases. Functional E-cadherin complexes are necessary for this effect. The relationship between increased LAR levels and activity of the EGF receptor is still unclear. It has been reported that EGF receptor signaling is suppressed at confluence. It is intriguing to speculate that the density dependent increase in LAR is involved in this suppression. We do not have data as yet to link these two processes. Finally, we have now developed a transgenic mouse with targeted expression of human LAR to the mammary gland. Characterization of mammary gland development and tendency to tumorigenesis will be pursued. This will be an important complement to our parellel studies with the LAR knockout mouse. These studies will commence shortly as Dr. Kisseberth establishes his laboratory at Ohio State.

References: NA

Appendices: None